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Further Studies on the Reaction of *Opalina* to Various Laboratory Culture Media.

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INTRODUCTION.

THE possibility of growing a parasite outside of its normal host is of the utmost importance in order to study that parasite and to determine its life cycle. During the last twenty years a large number of papers have appeared in which this phase of parasitological research has been emphasized. The technique and culture media have been improved to such an extent that the time does not seem far distant when all pathogenic protozoa can be grown outside of the host.

The intestinal flagellates of various animals, including man, have very readily responded to such treatment, and the first published reports deal more or less with this class of single-celled animals. About 1910 Dock and Bass successfully cultured the malarial parasite, but it was not until 1925 that the very interesting and important pathogenic sarcodine, *Endamœba histolytica* (*dysenteriæ*) was grown in an artificial medium by Bœck and Drbohlav ('25, 3). So successful was the culturing of this ameba on an especially prepared medium that the cultures in the first series of experiments retained their pathogenicity for seven months and more. The organisms were subcultured every forty-eight hours, and at the end of the seven months kittens inoculated with these amebas soon developed serious amebiasis. Different species of the parasitic ciliates have also been cultured. There is only one ciliate, *Balantidium coli*, pathogenic to man, and it has been reported as being successfully cultured. It is interesting to note that parasitic protozoa representing all classes of the phylum have finally been induced to grow and multiply on artificial media.

METHODS AND MATERIAL.

For some time in the Zoölogy Department at the University of Kansas some attention has been given to the study of the ciliate, *Opalina obtrigonoidea,* parasitic in the cloaca of the common leopard frog, *Rana pipiens.* In this series of experiments 166 *Rana pipiens* were examined, and of these 80 proved to be heavily enough parasitized for use.

A paper published by one of us (Larson '28, 6) contains a complete account of how the opalinas were obtained, how the media were made up, and how the cultures were prepared and examined. The same technique was followed in this series, and so the details will not be repeated.

It will suffice to mention that it is very necessary to use well sterilized culture dishes, instruments, etc. It is of the utmost importance to use care in handling these organisms in order to avoid contamination.

Several of the media used in the previous experiments were modified, and therefore those details will be explained. The media most often modified were Cleveland's, Pütter's, Locke's and Ringer's. To 75 cc. of Pütter's fluid was added 25 cc. of blood serum, or 90 cc. of the first and 10 cc. of the second. Lœffler's blood serum was used and was made up by using one gram of the dry powder to 100 cc. of distilled water heated to 42° C. Pütter's fluid was also modified by adding 250 milligrams of dextrose to 250 cc. of the medium. The main constituent of Pütter's medium is Rochelle salts and this was added to Locke's in the same proportion as given in the original formula for Pütter's fluid.

Locke's and Pütter's were also modified by the addition of egg albumen. To the well beaten white of one egg, 500 cc. of either medium was added and kept in a hot-water bath for 30 minutes. This preparation should be stirred occasionally and then filtered. Some cultures were set up somewhat differently from those reported in the previous paper, since in these experiments the entire absence of the cloacal wall and cloacal content was desired. All of these cultures correspond to those called "clear" in the previous experiments. The number of cultures made from the opalinas in one cloaca was dependent upon the relative number present. In short, each culture dish contained the medium and the opalinas free from fecal material, and most of the bacteria. The number of bacteria present can be very much reduced by passing the opalinas through several changes of the medium. This can easily be done by transferring the parasite from dish to dish by means of a capillary pipette.

DISCUSSION.

As suggested in the introduction, all classes of protozoa have been successfully cultured, nevertheless less work has been done on the ciliates than on any of the others. That was the first thing considered when *Opalina obtrigonoidea* was chosen for these experiments; secondly, this parasite is fairly easy to secure. A continuous supply of material is a very important factor in any line of investigation.

In the published paper (Larson, '28, 6), "Reaction of Opalinas to Various Laboratory Culture Media," the following conclusions were arrived at: (1) *Opalina obtrigonoidea* can be maintained outside of the host for various lengths of time, depending upon the culture medium used. (2) Pütter's fluid is the best of the culture fluids not having blood serum in the original formula. (3) Of the more common laboratory media Locke's fluid is best, with a 33⅓ per cent sea water a close second. (4) Of all the media tried in this series of experiments, a modified serum-saline-citrate proved to be the best for keeping opalinas in process of division and growth outside of their normal host. (5) Any one of the common laboratory media will probably prove to be more effective if serum is added. (6) When culturing with a fluid which does not contain blood serum, a piece of cloacal wall materially helps to increase the longevity of the opalinas in the culture.

Because of the efficacy of Cleveland's solution of serum-saline-citrate, it was decided to try adding blood serum or egg-albumen to some of the other laboratory maintaining fluids such as Locke's and Ringer's, also Pütter's. Pütter's fluid was developed by the German investigator, Pütter, in 1905, and has proven to be an excellent maintaining medium for opalinas. It was developed for the specific purpose of "growing opalinas." The food supply for these organisms is undoubtedly one of the prime factors entering into the problem. Allowing the cloacal content to remain in the culture seems to cause eventually too rapid a multiplication of bacteria. Our first work brought out conclusively the fact that the culture lived longest when there was none or very little of the cloacal content present but with a piece of the cloacal wall in it.

The addition of egg albumen or blood serum in certain definitely worked out proportions materially increased the longevity of the opalinas. This fact makes subculturing more simple because one is not dependent upon a supply of fresh cloacal wall when replenish-

ing the medium in the mass cultures or in making new cultures from the original ones. The following summary shows the results obtained.

Medium.	Increase in longevity, in hours.
Pütter's (90 cc.) and blood serum (10 cc.)	72
Pütter's and egg albumen	48
Locke's and egg albumen	24
Locke's (90 cc.) and blood serum (10 cc.)	24
Pütter's and dextrose	None
Ringer's and blood serum	None
Ringer's and egg albumen	None

These are the results from mass cultures where no subculturing was done. Fresh medium was occasionally added because there was some evaporation, in spite of the fact that the culture dishes were always covered.

In Pütter's fluid the opalinas maintained themselves in mass cultures and without subculturing for sixty-six hours. The addition of the blood serum increased the maintaining power of the medium seventy-two hours. In Locke's fluid the opalinas lived sixty hours, but with the addition of the blood serum to the medium its maintaining power was increased. For further details concerning other experiments with which comparisons are made in this table the reader is referred to an earlier paper (Larson '28, 3).

We regret that at present it is impossible to give a table of measurements for the individual opalinas after fission in the culture medium. (We expect to clear up this matter in the near future.) That would answer the question: Had there been actual growth of the daughter opalina after division? We felt that there must have been some increase in the total bulk of opalina protoplasm. We made numerous observations on opalinas in the process of fission and were quite confident that, owing to the favorable condition of the cultures, especially those that were maintained so long by subculturing the opalinas which had divided some hours previously were not noticeably smaller than opalinas which had not divided.

Some cultures were subcultered every second or third day. This was more or less dependent upon the condition of the culture. The following sample protocols will serve as illustrations for a great number of experiments worked out in the course of this investigation.

A. Pütter's medium (90 cc.) and serum (10 cc.).

March 9, original culture divided into six cultures.
March 11, each culture was subdivided.
March 13, each culture was subdivided.
March 15, each culture was subdivided.

On March 18 culture C of this series of six cultures was in excellent condition, and division had been so rapid that on this date it was divided into four new cultures with fresh medium added. The following is a history of these cultures:

I. Subcultured March 21. All opalinas dead March 23.

II. Not subcultured. Almost all opalinas dead March 23. Culture was destroyed.

III. Subcultured March 20, 22, 24. Almost all opalinas dead March 24. Culture was destroyed.

IV. Subcultured March 23, 25, 27. On the 29th so few opalinas were living that the culture was destroyed.

B. Pütter—Egg medium.

Four cultures made from original culture April 7.

Each culture had the following history: Subcultured April 9, 12, 14, 16, 18, 19, 23, 25.

On April 14 the C culture of these four cultures was so loaded with opalinas that three new cultures were put together in fresh medium and this culture lasted until April 30.

In all of these experiments where subculturing was done, it is understood to mean the transfer of all the opalinas by a capillary pipette from the old culture medium into a well sterilized watch glass filled with fresh medium of the kind used in the set.

An interesting condition was noticed in one set of cultures where a modified Cleveland's medium had been used. This modification was a 0.1 per cent of sodium citrate instead of 1 per cent as used by Cleveland. The rate of fission was apparently stimulated to an abnormal degree. In the springtime the division of opalinas is normally quite rapid, and it was in that season that these conditions were observed, but this division was very different from all other divisions which we observed macroscopically. Before the parent opalina had completed its fission each of the daughter opalinas had begun division. This peculiar condition was carried to such an extreme that the dividing opalinas presented the appearance of a ball composed of eight or more individuals, none of them completely separated. In some cases they looked like the spokes of a wheel, radiating from a common center. Someone not acquainted with the cultures might have called these dividing opalinas colonial protozoa. We hope to be able to repeat this experiment and to arrive at some conclusion concerning the exact cause of this peculiar condition.

It has been considered very important by several investigators that the media used for maintaining opalinas be made oxygen-free or nearly so. We found it possible, however, to grow opalinas fairly successfully without removing the oxygen from the media. Several of the media were boiled in the process of preparation, all media

were kept tightly corked, all cultures well covered, and special precautions were taken in making the transfers. This tended toward eliminating oxygen.

Dr. A. A. Schaeffer, of the Department of Zoölogy at the University of Kansas, offered many helpful suggestions during the course of these experiments.

CONCLUSIONS.

1. The addition of egg albumen or blood serum to any of the more generally used laboratory media will increase considerably the maintaining power of the media.

2. Pütter's medium with the addition of blood serum was found to be the best maintaining fluid. The addition of the egg albumen did not apparently increase its powers for longevity to such a great extent as the serum, but it did increase it beyond straight Pütter's.

3. The maintaining power of Locke's medium is increased several hours by the addition of blood serum or egg albumen.

4. The addition of either egg albumen or blood serum does away with the necessity of retaining the cloacal wall or content in the culture. This undoubtedly reduces the bacterial count, a very necessary factor to consider in any kind of culture work.

5. Subculturing the opalinas makes it possible to keep a culture a month or more for study. It is best to subculture systematically every 24 to 48 hours, depending upon the condition of the culture.

BIBLIOGRAPHY.

1. BOYD, MARK F. 1918. A note on the cultivation of trichomonas intestinalis. Jour. of Parasit, Vol. 6, 168-170.
2. BARRETT, H. P. and NANCY YARBROUGH. 1921. A method for the cultivation of *Balantidium coli*. Amer. Jour. of Trop. Med., Vol. 1, 161-164.
3. BŒCK, WM. C. and J. DRBOHLAV. 1925. The cultivation of *Endamœba histolytica*. Amer. Jour. of Hygiene, Vol. 5.
4. CLEVELAND, L. R. 1925. Toxicity of oxygen for Protozoa in vivo and in vitro; animals defaunated without injury. Biol. Bull., Vol. 48, 455-468.
5. HOGUE, M. J. 1921. *Waskia intestinalis*, its cultivation and cyst formation. Jour. Amer. Med. Assn., Vol. 77, 112-113.
6. LARSON, MARY E. 1928. Reaction of opalinas to various laboratory media. Tran. Amer. Micro. Soc., Vol. 47, 1-10.
7. PÜTTER, A. 1905. Die Atmung der Protozoen. Zeitschr. f. Allgem. Physiol., Vol. 5, 566-612.
8. TYLER, R. A. 1926. The cultivation of Opalina. Science, Vol. 54, 383-384.